



Genetic Similarity of Gonococcal Homologs to Meningococcal Outer Membrane Proteins of Serogroup B Vaccine

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ABSTRACT The human pathogens *Neisseria gonorrhoeae* and *Neisseria meningitidis* share high genome identity. Retrospective analysis of surveillance data from New Zealand indicates the potential cross-protective effect of outer membrane vesicle (OMV) meningococcal serogroup B vaccine (MenZB) against *N. gonorrhoeae*. A licensed OMV-based MenB vaccine, MenB-4C, consists of a recombinant FHbp, NhbA, NadA, and the MenZB OMV. Previous work has identified several abundantly expressed outer membrane proteins (OMPs) as major components of the MenB-4C OMV with high sequence similarity between *N. gonorrhoeae* and *N. meningitidis*, suggesting a mechanism for cross-protection. To build off these findings, we performed comparative genomic analysis on 970 recent *N. gonorrhoeae* isolates collected through a U.S. surveillance system against *N. meningitidis* serogroup B (NmB) reference sequences. We identified 1,525 proteins that were common to both *Neisseria* species, of which 57 proteins were predicted to be OMPs using *in silico* methods. Among the MenB-4C antigens, NhbA showed moderate sequence identity (73%) to the respective gonococcal homolog, was highly conserved within *N. gonorrhoeae*, and was predicted to be surface expressed. In contrast, the gonococcal FHbp was predicted not to be surface expressed, while NadA was absent in all *N. gonorrhoeae* isolates. Our work confirmed recent observations (E. A. Semchenko, A. Tan, R. Borrow, and K. L. Seib, Clin Infect Dis, 2018, <https://doi.org/10.1093/cid/ciy1061>) and describes homologous OMPs from a large panel of epidemiologically relevant *N. gonorrhoeae* strains in the United States against NmB reference strains. Based on our results, we report a set of OMPs that may contribute to the previously observed cross-protection and provide potential antigen targets to guide the next steps in gonorrhea vaccine development.

IMPORTANCE Gonorrhea, a sexually transmitted disease, causes substantial global morbidity and economic burden. New prevention and control measures for this disease are urgently needed, as strains resistant to almost all classes of antibiotics available for treatment have emerged. Previous reports demonstrate that cross-protection from gonococcal infections may be conferred by meningococcal serogroup B (MenB) outer membrane vesicle (OMV)-based vaccines. Among 1,525 common proteins shared across the genomes of both *N. gonorrhoeae* and *N. meningitidis*, 57 proteins were predicted to be surface expressed (outer membrane proteins [OMPs]) and thus preferred targets for vaccine development. The majority of these OMPs showed high sequence identity between the 2 bacterial species. Our results provide valuable insight into the meningococcal antigens present in the current OMV-containing MenB-4C vaccine that may contribute to cross-protection against gonorrhea and may inform next steps in gonorrhea vaccine development.

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Neisseria gonorrhoeae and *Neisseria meningitidis* are obligate human pathogens that are genetically closely related, sharing between 80 and 90% genome sequence identity (1–3). However, infections with these pathogens typically have very different clinical presentations (4). *N. gonorrhoeae* is responsible for gonorrhea, one of the most common bacterial sexually transmitted diseases (STDs), frequently causing inflammation of the urogenital tract. *N. meningitidis* infections can lead to life-threatening bacterial meningitis and septicemia (5) following penetration of the bacteria through the blood-brain barrier and colonization of the meninges.

Gonorrhea results in substantial morbidity and economic burden globally, with an estimated 100 million cases worldwide. In the United States, gonorrhea is the second most common notifiable disease. More than 550,000 infections were reported to the U.S. Centers for Disease Control and Prevention (CDC) in 2017, the highest number since 1991 (6, 7). Though *N. gonorrhoeae* infections typically trigger urethritis in men and cervicitis in women, mucosal infections of the rectum, pharynx, and eye are also common (8–10). Gonorrhea facilitates human immunodeficiency virus (HIV) transmission through the stimulation of antiapoptotic proteins essential for the HIV life cycle (11). The emergence of *N. gonorrhoeae* strains that are resistant to nearly all classes of antibiotics available for treatment and the lack of an effective gonococcal vaccine (9, 10, 12, 13) underscore the urgent need for new prevention and control measures.

In contrast to polysaccharide-based vaccines targeting *N. meningitidis* serogroups A, C, W, and Y, protein-based vaccines have been developed for *N. meningitidis* serogroup B (NmB) (14, 15). NmB capsular polysaccharides contain polysialic acid structures that are similar to human neuronal glycoproteins (16); this results in poor immunogenicity and poses the risk of triggering an autoimmune response. Vaccines containing NmB outer membrane vesicles (OMVs) have been utilized in a number of countries, where they are primarily designed to match outbreak strains circulating during regional epidemics (17, 18). Previous studies suggested a decline in gonorrhea cases following the introduction of meningococcal serogroup B (MenB) OMV vaccines. Retrospective analyses of surveillance data in Cuba, Norway, and New Zealand have indicated potential cross-protection from MenB OMV vaccines against *N. gonorrhoeae* infections (19–21). A case-control study in New Zealand estimated that vaccinated individuals had a 31% lower risk of developing gonorrhea (20); a decision-analysis model showed a 20% assumptive reduction in gonorrhea due to the cross-protection conferred by the licensed protein-based and OMV-containing MenB-4C (Bexsero) vaccine. Recently, Semchenko and colleagues analyzed the sequence identity and conservation of MenB-4C vaccine antigens against publicly available gonococcal genomes and demonstrated the potential contribution of OMV components to the observed cross-protection (22). Decreased gonorrhea incidence could lower morbidity and rates of complications, such as HIV (23), and reduce the risk of the emergence of untreatable antibiotic-resistant gonorrhea.

MenB OMV vaccines contain outer membrane proteins (OMPs), with porin A (PorA) being immunodominant (24). However, evidence suggests that the *porA* gene might only be present as a pseudogene in *N. gonorrhoeae* and is not expressed (25, 26). Therefore, the mechanism of action for protective immunity conferred by the OMV vaccines against *N. gonorrhoeae* has yet to be fully explained. Depending on the bacterial species and detection methods used to assess them, OMVs may comprise more than 300 different protein components, of which up to 80% are OMPs (27). In addition to PorA, meningococcal OMPs such as PorB, FetA, and OpcA have been shown to induce protective antibody responses (18, 28, 29). Nevertheless, other OMPs and/or membrane-exposed antigens also potentially generate cross-reactivity inducing broader

protection. Numerous proteins have been extracted and identified from NmB OMVs (30–35).

Reverse vaccinology, which includes genome and proteome mining, has been utilized to identify potentially surface-exposed antigens that are highly immunogenic and protective for vaccine development (36–38). The multicomponent MenB-4C vaccine contains the following 3 immunogenic antigens identified by reverse vaccinology: factor H binding protein (FHbp) fused with GNA2091, *Neisseria* adhesion A (NadA), and *Neisseria* heparin binding antigen (NhbA) fused with GNA1030 supplemented with the OMV of the New Zealand epidemic strain (NZ98/254) to warrant broader immunogenicity (39). Although the formulation is not identical to that of the New Zealand MenB OMV (MeNZB) vaccine, immunogenic OMV components included in the MenB-4C vaccine may cross-protect against *N. gonorrhoeae* infections.

In this study, we sequenced and analyzed a diverse genome data set of 970 *N. gonorrhoeae* isolates collected from Gonorrhea Isolate Surveillance Project (GISP) sites across the United States between 2014 and 2016. We identified common proteins present in both *N. gonorrhoeae* and *N. meningitidis* and assessed the diversity of MenB vaccine antigens and OMV proteins to and within the U.S. *N. gonorrhoeae* strain collection. Bioinformatic tools were applied to predict the subcellular localization of each identified common protein. We found a significant number of predicted OMPs present in both *Neisseria* species, which could potentially contribute to cross-protection against gonorrhea and may serve as candidate antigens for gonococcal vaccine development.

RESULTS

Identification of common proteins and their subcellular localizations. *N. gonorrhoeae* and *N. meningitidis* show the closest genetic relationship within the *Neisseria* family (1–3, 40). In total, we identified 1,525 proteins that were common among our set of 970 *N. gonorrhoeae* genomes and 4 NmB references, including MenB-4C vaccine antigens and major abundant proteins previously found in OMVs (see Table S1 in the supplemental material). Using various *in silico* prediction tools, we predicted the subcellular localization of each common protein found in both *Neisseria* species. The outer membrane localization was first determined for proteins in NmB and then compared them to those of the respective gonococcal homologs. A protein received its outer membrane-associated assignment only after being confirmed by at least 2 of 3 prediction tools, along with the detection of a signal peptide. A schematic workflow of the OMP identification process is shown in Fig. 1.

Among the major MenB-4C vaccine antigens, only FHbp and NhbA with their corresponding fusion proteins (GNA2091 and GNA1030, respectively) were found in all 4 NmB strains and 970 *N. gonorrhoeae* isolates (Table 1). A full-length NadA protein was present in the MC58 and CU385/83 NmB strains, but it was truncated in the other two NmB strains (H44/76 and NZ98/254) and absent in all *N. gonorrhoeae* isolates used in this study. In contrast to the NmB FHbp, the gonococcal FHbp homolog was predicted as not being expressed on the cell surface, based on its subcellular localization and the lack of a signal peptide (Table 1). The gonococcal NhbA was predicted as being surface exposed. Both fusion proteins (GNA2091 and GNA1030) were predicted as being surface exposed in *N. meningitidis*; GNA1030 was surface exposed in *N. gonorrhoeae*, while only 1 tool predicted GNA2091 to be surface exposed in *N. gonorrhoeae*.

While the *porA* gene was identified in 966 of 970 (99.5%) *N. gonorrhoeae* isolates, the DNA sequences contained inactivating mutations in the promoter region (Fig. 2), along with frameshift deletions in the coding sequence resulting in premature internal stops, consistent with previous reports describing *porA*'s pseudogene status in *N. gonorrhoeae* (25, 26).

Common outer membrane proteins shared between NmB and *N. gonorrhoeae* strains. Among the 1,525 common proteins, 57 were predicted to be OMPs (Table 2); 28 of these proteins had known biological functions, while the remaining 29 were either putative or hypothetical proteins. Of the 57 predicted OMPs, we identified 25

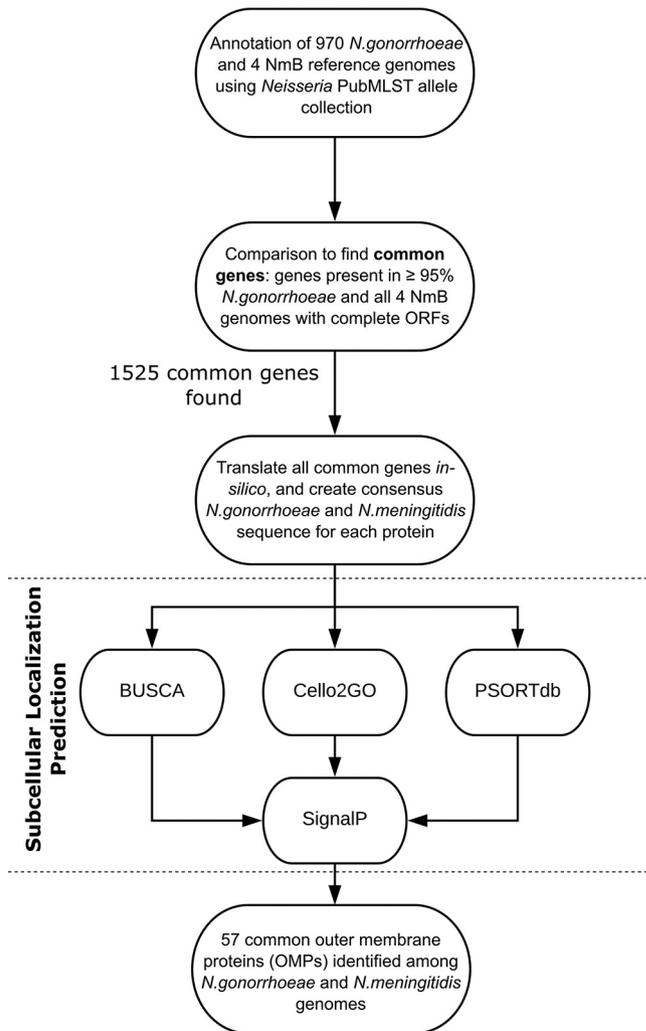


FIG 1 Schematic presentation of common protein identification and assessment of proteins' subcellular localization. Ng, *N. gonorrhoeae*; Nm, *N. meningitidis*.

proteins that had been previously detected in the NZ98/254 OMV components using different laboratory approaches (24, 30, 32, 34), 12 of which were abundantly and consistently expressed across different OMV lots (32) (Table 2). These 12 proteins include some major immunogenic antigens that have been tested experimentally as potential gonorrhea vaccine candidates, such as PorB, PilQ, OpcA, and Omp85 (Bama)

TABLE 1 Sequence analysis and subcellular localization of MenB-4C vaccine antigens^a

Vaccine antigen	NEIS no.	NmB strains (n = 4)		<i>N. gonorrhoeae</i> strains (n = 970)	
		No. of isolates with full-length protein	Predicted OMP	No. of isolates with full-length protein	Predicted OMP
FHbp	NEIS0349	4	+	970	-
NhbA	NEIS2109	4	+	970	+
NadA	NEIS1969	2	+	0	NA
OMV (PorA)	NEIS1364	4	+	0	NA
GNA2091	NEIS2071	4	+	970	+/-
GNA1030	NEIS1183	4	+	970	+

^aCellular localization and signal peptide predictions were determined using the *in silico* tools BUSCA, Cello2GO, PSORTdb, and SignalP. OMP, outer membrane protein; +, predicted as OMP by at least 2 tools; -, not predicted as OMP by any of the tools; +/-, predicted as OMP by 1 tool; NA, not applicable due to the absence of an ORF.

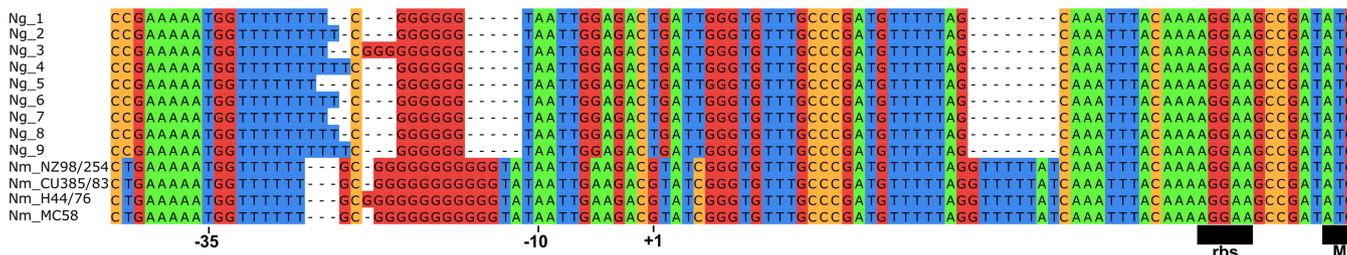


FIG 2 The *porA* promoter region for *N. gonorrhoeae* and *N. meningitidis*. The putative meningococcal -10 and -35 locations as well as the ribosome binding site and the start codon are indicated. The *porA* promoter sequences for all 970 *N. gonorrhoeae* genomes were filtered down to 9 unique sequences. The bottom four rows consist of the *porA* promoter sequences for the *N. meningitidis* references. The gaps represent nucleotides that were missing from the respective sequence compared to the rest.

(9, 12, 13, 41). The remaining 34 OMPs, including the MenB-4C vaccine antigens FHbp and Nhba, had not been previously identified in NZ98/254 OMVs. Two OMV proteins (MtrE and NEIS1428) were predicted as being outer membrane exposed in *N. meningitidis*; however, a signal peptide was detected only by 1 of the prediction tools. A similar observation was also seen for these 2 OMV proteins in *N. gonorrhoeae*.

Of note, a previous report predicted the meningococcal PilE (NEIS0210) as an OMP (32); however, the prediction tools used in our study indicated extracellular/periplasmic/plasma membrane localization of PilE and absence of a signal peptide in both *N. meningitidis* and *N. gonorrhoeae*. In addition, a gene coding for OMP P1 (NEIS0073) contained an internal stop codon in all 970 *N. gonorrhoeae* isolates. While *mafA* (NEIS0596) was present in the 4 NmB and 970 *N. gonorrhoeae* genomes, it was fragmented across multiple contigs within the NZ98/254 genome. Furthermore, Tbp1 (NEIS1690) was only found in 89% of all *N. gonorrhoeae* strains, which did not meet our threshold of 95% for common protein selection. Therefore, PilE, OMP P1, MafA, and Tbp1 were not included in our list of common proteins and/or OMPs.

Sequence similarity of OMPs found within NmB and *N. gonorrhoeae* strain collections. Next, we analyzed the genetic similarity of each of the 57 predicted OMPs between the two *Neisseria* species and within the whole *N. gonorrhoeae* collection to assess sequence conservation. To this end, we focused our analysis on the NZ98/254 strain, from which the OMV in the MenB-4C vaccine derives. It has been shown that vaccines against Japanese encephalitis virus may induce cross-reactivity to West Nile virus and dengue virus since their common proteins share approximately 80% and 50% amino acid sequence homology, respectively (42, 43). Based on this knowledge, we used a minimum sequence similarity of 80% as a threshold for potential cross-protection against *N. gonorrhoeae* (Fig. 3). The mean sequence similarity between *N. meningitidis* and *N. gonorrhoeae* for all 57 OMPs was $91.0\% \pm 9.8\%$. At the amino acid level, 50 of 57 (88%) OMPs from the NZ98/254 strain showed 83 to 99% sequence

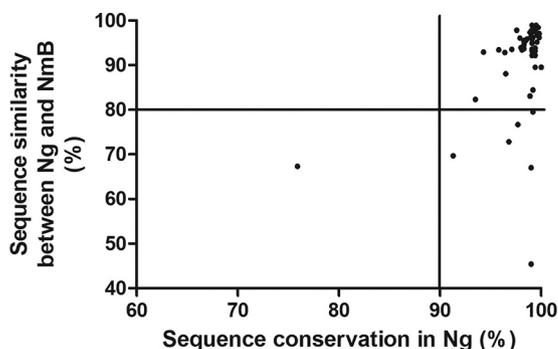


FIG 3 Amino acid sequence similarity of 57 common OMPs found in 970 *N. gonorrhoeae* and NmB (NZ98/254) strains. A value of 80% was set as a minimum level of sequence similarity that might confer cross-protection. The majority of the common OMPs had $>90\%$ sequence conservation in *N. gonorrhoeae*.

TABLE 2 Predicted outer membrane proteins and sequence similarity in *N. gonorrhoeae* and NmB strains

NEIS no.	Protein name ^a	Identified in OMVs	Amino acid sequence similarity (%)				Experimentally tested as GC vaccine ^c
			Between <i>N. gonorrhoeae</i> and NZ98/254 ^b		Within <i>N. gonorrhoeae</i>		
			Range	Mean ± SD	Range	Mean ± SD	
NEIS2109	NhbA		67.5–88	72.8 ± 5.2	78.3–100	96.8 ± 5.0	
NEIS1183	GNA1030		90.9–93.5	92.2 ± 0.9	98.4–100	99.1 ± 0.5	
NEIS2020	PorB	+	66.2–94.2	69.7 ± 2.9	65.2–100	91.3 ± 6.2	+
NEIS1963	FetA	+	90.5–95	92.8 ± 1.4	89.5–100	96.4 ± 1.9	
NEIS0408	PilQ	+	89.3–97.2	93.1 ± 2.3	95.7–100	99.2 ± 0.6	+
NEIS0173	Omp85 (BamA)	+	95–95.8	95.2 ± 0.2	99–100	99.6 ± 0.2	+
NEIS1783	RmpM	+	92.6–93.6	93.2 ± 0.3	98.7–100	99.4 ± 0.3	
NEIS2198	OpcA	+	44.8–47.1	45.4 ± 0.6	97.7–100	99 ± 0.5	+
NEIS0612	NspA	+	90.8–95.7	94 ± 0.9	92–100	98.2 ± 1.5	+
NEIS1632	MtrE	+	93–97.5	94.9 ± 1.7	95–100	99.1 ± 1.3	+
NEIS0586	MafB1	+	79–80.9	79.5 ± 0.4	96–100	99.2 ± 1.1	
NEIS1917	MetQ	+	96.5–99.3	96.9 ± 0.7	97.2–100	99.6 ± 0.9	+
NEIS0653	ComL	+	98.1–99.6	98.4 ± 0.4	97.8–100	99.4 ± 0.6	
NEIS1687	OM phospholipase A precursor	+	98.4–98.9	98.6 ± 0.1	99.2–99.7	99.5 ± 0.2	
NEIS1468	LbpA	+	86.3–96.3	95.5 ± 1.8	87.9–100	98.3 ± 2.4	+
NEIS1487	FkpA (macrophage infectivity protein)	+	96.7–99.2	97.3 ± 0.6	96–100	98.9 ± 1.0	
hmbR	HmbR		89.5–89.6	89.5 ± 0.1	100–100	100 ± 0.0	
NEIS0566	LolB		98.1–98.5	98.4 ± 0.2	99–100	99.7 ± 0.3	
NEIS1418	NlpD	+	90.3–94	93.1 ± 0.7	95.4–100	99.2 ± 0.7	
NEIS1462	H.8 OMP		95.1–98.3	97.8 ± 0.8	88.6–100	97.6 ± 3.8	
NEIS1933	VacJ-related protein		94.9–95.6	95.3 ± 0.2	95.6–100	98.4 ± 1.6	
NEIS0338	Ferric siderophore receptor protein		74.9–97.2	92.9 ± 7.5	76.6–100	94.3 ± 8.8	
NEIS1813	OstA		93.2–94.3	93.5 ± 0.4	98.9–100	99.2 ± 0.3	
NEIS1691	TbpB		60.8–86.3	67.3 ± 5.3	58.9–100	75.9 ± 7.7	+
NEIS1549	AniA		83.4–92.3	84.4 ± 2.4	95.8–100	99.2 ± 0.6	+
NEIS1205	Ape1		94.5–97.4	95.3 ± 0.6	95.2–100	98.3 ± 1.3	
NEIS2124	Lipoprotein		98.6–99.3	98.9 ± 0.3	98.6–100	99.1 ± 0.4	
NEIS1063	Putative periplasmic protein	+	96.3–96.7	96.6 ± 0.2	99.1–100	99.7 ± 0.3	
NEIS1066	Putative periplasmic protein	+	93.3–94.1	93.8 ± 0.3	98.7–100	99.4 ± 0.3	
NEIS0275	Putative OM solvent tolerance protein (LPS assembly protein)	+	89.1–89.9	89.5 ± 0.2	92.6–100	99.4 ± 1.1	
NEIS0944	Putative OM receptor protein (TonB-dependent receptor)	+	96.7–97.3	96.9 ± 0.1	99.4–100	99.7 ± 0.1	
NEIS1428	Putative OM substrate binding protein (TonB-dependent receptor)	+	94.8–96.5	95.8 ± 0.4	97–100	98.6 ± 0.6	
NEIS0739	Putative amino acid permease substrate-binding protein	+	97.1–98.5	97.8 ± 0.3	98.6–100	99.4 ± 0.4	
NEIS1920	Putative transglycosylase	+	95.9–97.8	97.4 ± 0.3	96.3–100	99.1 ± 0.6	
NEIS2112	Putative OMP		98.4–99.1	98.9 ± 0.2	98.1–100	99.5 ± 0.4	
NEIS0261	Putative periplasmic protein		86.2–97	93.5 ± 2.8	85.4–100	97.1 ± 3.7	
NEIS0504	Putative thiamine biosynthesis protein		96–96.3	96.2 ± 0.1	99.4–100	99.8 ± 0.2	
NEIS0630	Putative periplasmic protein		92.1–92.6	92.2 ± 0.2	99–100	99.4 ± 0.3	
NEIS0729	Putative secreted protein		91.4–96.3	93.9 ± 1.3	92.9–100	98 ± 1.9	
NEIS1304	Putative membrane lipoprotein		96.7–97.2	97.1 ± 0.2	99.5–100	99.8 ± 0.3	
NEIS1195	Putative peptidyl-prolyl <i>cis-trans</i> isomerase A		92.4–94.5	93.6 ± 0.6	97.3–100	99.1 ± 0.7	
NEIS1935	Putative periplasmic transport protein		91.8–92.8	92.2 ± 0.4	98.5–100	99.1 ± 0.4	
NEIS0196	Putative OM lipoprotein	+	92.8–95.2	93.4 ± 0.9	91.2–99.2	95.8 ± 3.6	
NEIS1084	Putative periplasmic protein	+	93.1–95	93.7 ± 0.6	97–100	98.3 ± 0.8	
NEIS1125	Putative periplasmic protein		95.3–97	95.8 ± 0.6	98.8–100	99.1 ± 0.3	
NEIS1172	Putative periplasmic protein		92.7–99.7	93.2 ± 1.1	92.7–100	99.1 ± 1.4	

(Continued on following page)

TABLE 2 (Continued)

NEIS no.	Protein name ^a	Identified in OMVs	Amino acid sequence similarity (%)						Experimentally tested as GC vaccine ^c
			Between <i>N. gonorrhoeae</i> and NZ98/254 ^b		Within <i>N. gonorrhoeae</i>		Mean ± SD		
			Range	Mean ± SD	Range	Mean ± SD			
NEIS1367	Putative lipoprotein		96.1–96.6	96.2 ± 0.3	98.9–100	99.2 ± 0.4			
NEIS1271	Hypothetical protein	+	95.5–97.1	96.4 ± 0.4	97.3–100	99 ± 0.5			
NEIS1858	Hypothetical protein		92.4–99.1	94.5 ± 1.5	91.6–100	98.3 ± 1.9			
NEIS1945	Hypothetical protein		74.2–99.2	82.3 ± 8.3	74.0–100	93.5 ± 9.2			
NEIS1485	Hypothetical protein		97.3–100	97.7 ± 0.7	97.3–100	99 ± 0.9			
NEIS1546	Hypothetical protein		88.4–97.7	96.1 ± 2.2	87.0–100	97.9 ± 3.4			
NEIS1746	Hypothetical protein		62.7–87.7	67 ± 9.3	98.4–100	99 ± 0.5			
NEIS2446	Hypothetical protein		83.5–91.2	88.1 ± 2.4	90.1–100	96.5 ± 2.7			
NEIS0790	Hypothetical protein		74.6–80.6	76.7 ± 2.2	93–100	97.7 ± 2.6			
NEIS1136	Hypothetical protein		82.6–83.7	83.1 ± 0.5	97.8–100	98.9 ± 0.6			
NEIS1253	Hypothetical protein		91.9–97.5	93.4 ± 1.3	93.8–100	98.1 ± 1.5			

^aPreviously reported proteins from detergent-extracted NZ98/254 outer membrane vesicles (OMVs) (24, 30, 32, 34). Proteins in boldface are most abundantly expressed, and their expression was not significantly expressed across different OMV lots (32). OM, outer membrane; LPS, lipopolysaccharide; OMP, outer membrane protein.

^bValues in boldface show mean sequence similarity that is greater than 80%.

^cElicits bactericidal antibodies to *N. gonorrhoeae* (9, 12, 13, 41). GC, gonorrhea.

similarity to all 970 *N. gonorrhoeae* isolates, of which 24 proteins had previously been identified in NZ98/254 OMVs (Table 2). The remaining 7 OMPs had 45 to 79% sequence similarity to *N. gonorrhoeae*.

Among MenB-4C vaccine antigens, FHbp of the NZ98/254 strain showed a modest mean amino acid sequence similarity to *N. gonorrhoeae* (64%); the NhbA similarity was higher (73%) (Table 2). The 2 fusion proteins of NZ98/254 had high sequence similarity to *N. gonorrhoeae*, at 95% for GNA2091 and 92% for GNA1030. These 4 vaccine antigens were highly conserved (97 to 99% identity) within the *N. gonorrhoeae* collection. Of all the predicted OMPs, OpcA of NZ98/254 had the lowest mean similarity to *N. gonorrhoeae* (45%), followed by the abundant OMV protein PorB (70%), but the 2 proteins showed 99% and 91% sequence conservancy within *N. gonorrhoeae*, respectively (Table 2).

The majority (56 of 57) of predicted OMPs displayed 91 to 100% sequence conservation within the 970 *N. gonorrhoeae* isolates, with the lowest mean similarity observed for TbpB (76%) (Table 2). It is noteworthy that the sequence similarities at the amino acid and DNA levels were consistent between NZ98/254 and all *N. gonorrhoeae* strains, except for PorB, PilQ, and OpcA, which had ~10% differences (Table S2). Other major OMV proteins, such as FetA, PilQ, Omp85 (BamA), RmpM, and MtrE of NZ98/298 consistently showed $\geq 93\%$ mean amino acid sequence similarity to and within the *N. gonorrhoeae* isolates. The NZ98/298 nitrite reductase AniA, essential for *N. gonorrhoeae* anaerobic respiration and biofilm formation (44–46), showed 84% and 99% sequence similarity to and within *N. gonorrhoeae* isolates, respectively (Table 2).

Phylogenetic analysis was also conducted to assess the sequence diversity of the OMPs found within the two *Neisseria* species. Six common OMPs that had previously been identified in the NZ98/254 OMV and showed amino acid sequence similarity above the 80% cutoff to and within the 970 *N. gonorrhoeae* strains were selected for this purpose. These OMPs were FetA, PilQ, Omp85 (BamA), RmpM, NspA, and MtrE. Our analysis indicated that PilQ, Omp85 (BamA), RmpM, NspA, and MtrE of NZ98/254 formed a distinct cluster from the other 3 NmB strains, whereas FetA of CU385/83 was distinct from the rest of the NmB group (Fig. 4). This pattern of distinction among the NmB strains was also found with other OMV proteins, such as FHbp and its fusion protein (GNA2091), NhbA, AniA, and OpcA (Fig. S1), in contrast to PorB, which formed one cluster. Consistent with the number of unique alleles, RmpM, NspA, and MtrE formed clusters that are less diverse within *N. gonorrhoeae* than other OMPs, particularly FetA and PorB (Fig. 4 and S1). It is noteworthy that 2 alleles of gonococcal FetA, 1 allele of NhbA, and PorB shared a cluster with the NmB ones, suggesting that these alleles are closely related.

DISCUSSION

Increasingly resistant *N. gonorrhoeae* strains represent a global threat, and no gonococcal vaccine is currently available (13). Two vaccine candidates tested in a full cycle of field trials in humans, a whole-cell vaccine within the Aboriginal population of Inuits in northern Canada (47) and a pilus-based vaccine for high-risk U.S. military personnel stationed in South Korea (48), were unsuccessful due to a lack of efficacy compared to placebo-treated groups. Evidence for potential cross-protection against gonorrhea conferred by MenB OMV vaccines (19–21) could reduce the disease burden. Our analysis of NmB and *N. gonorrhoeae* sequences revealed more than 1,500 common proteins shared across the genomes of these 2 *Neisseria* species. We evaluated antigens of the MenB-4C vaccine, a licensed protein-based vaccine containing OMVs of the New Zealand *N. meningitidis* epidemic strain (NZ98/254) (32, 34), to assess its potential cross-protective effect, particularly on the recent U.S. gonococcal strains.

Major MenB-4C vaccine antigens FHbp, NadA, and PorA are less likely to contribute to cross-protection against *N. gonorrhoeae* infections. The gonococcal FHbp has previously been shown to not be surface expressed and is unable to bind factor H (49). A recent study demonstrates that antibodies raised against the 3 major MenB-4C antigens did not recognize FHbp in whole-cell lysates of *N. gonorrhoeae* test strains (22), which

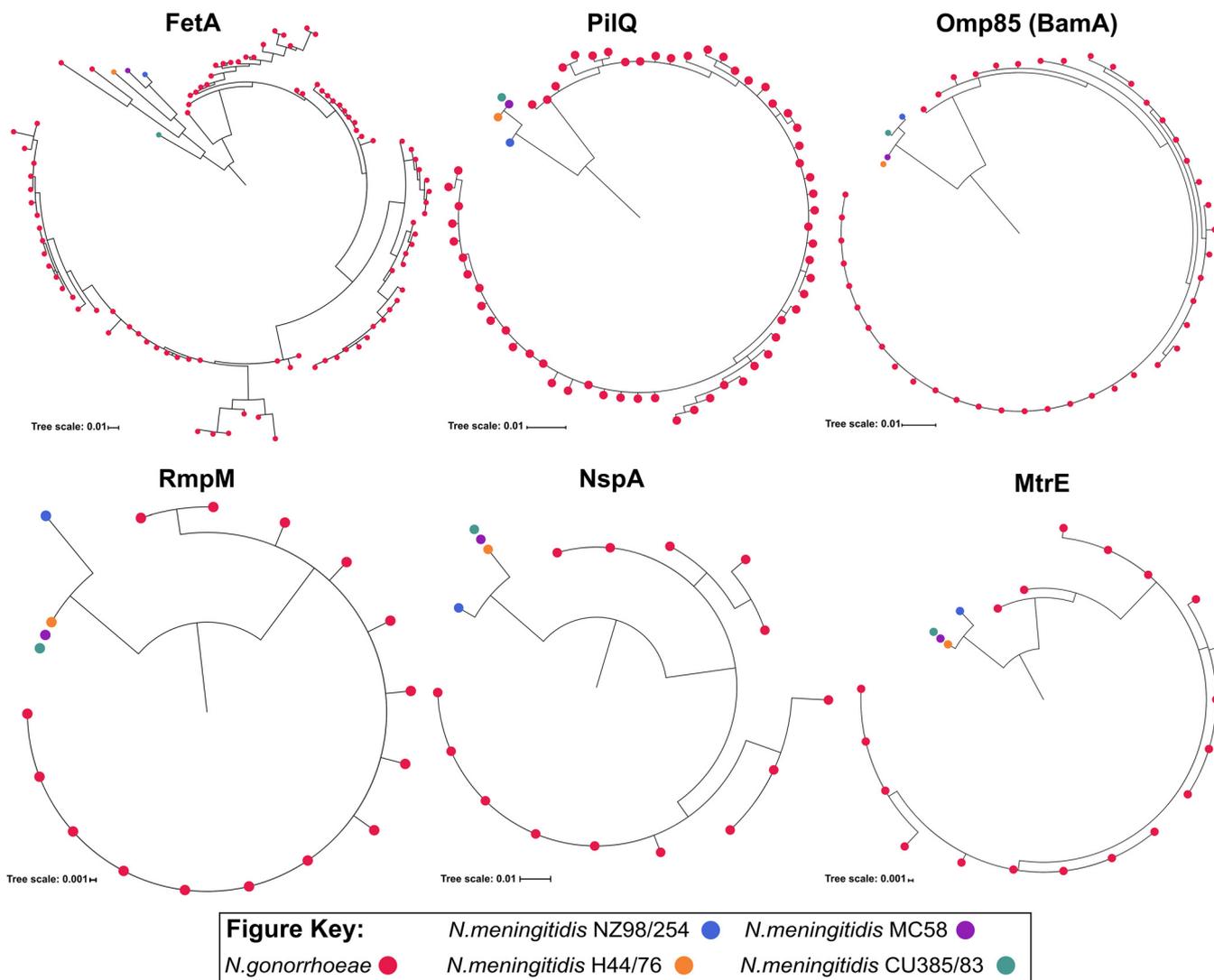


FIG 4 Phylogenetic clustering of FetA, PilQ, Omp85 (BamA), RmpM, NspA, and MtrE proteins found in 4 NmB and 970 *N. gonorrhoeae* strains. Each red dot represents sequences obtained from a unique *N. gonorrhoeae* allele. In total, 16 unique alleles were found within NmB strains, as follows: 4 for FetA, 3 for PilQ, 3 for Omp85 (BamA), 2 for RmpM, 2 for NspA and 2 for MtrE; there were 248 unique alleles within *N. gonorrhoeae* strains, as follows: 95 for FetA, 61 for PilQ, 44 for Omp85 (BamA), 15 for RmpM, 14 for NspA, and 19 for MtrE.

confirms that the gonococcal FHbp homolog is not expressed on the cell surface. Furthermore, in accordance with previous reports (2, 25, 26), NadA was absent in all 970 *N. gonorrhoeae* isolates analyzed in this study, while *porA*'s promoter appeared to be inactive for DNA transcription. Conversely, Nhba showed moderate amino acid sequence similarity to *N. gonorrhoeae* strains, as shown by others (2, 22), and may therefore contribute to cross-protection to an extent. Similar levels of sequence similarity were found for Nhba of the other 3 NmB strains, including MC58, consistent with the previous finding (2). Interestingly, we found a high level of sequence similarity between NZ98/254 and *N. gonorrhoeae* for the 2 recombinant fusion proteins GNA2091 and GNA1030. These fusion proteins were shown to induce protective immunity in mice (50) and may therefore exert a cross-protective effect. A recent report showed that antisera raised against recombinant MenB-4C vaccine antigens recognized the gonococcal homolog of Nhba and the 2 fusion proteins (22). However, only Nhba is surface expressed in *N. gonorrhoeae*, while GNA2091 and GNA1030 are not (22), consistent with previous findings (51, 52). Our analysis predicted GNA1030 to be surface exposed in both *N. meningitidis* and *N. gonorrhoeae*, suggesting that laboratory experiments are required to confirm the bioinformatic data.

In pathogenic bacteria, OMPs present important virulence determinants through their direct interaction with the host immune system (especially OMPs involved in adhesion and invasion) and have therefore become major targets for vaccine development (53, 54). Our sequence and subcellular localization analyses detected 57 common OMPs that were present in both NmB and 970 U.S. *N. gonorrhoeae* strains used in this study. One of the factors that has hampered gonorrhea vaccine development is the high antigenic variability of surface proteins among gonococcal strains (9, 12, 41). Our results demonstrate that FHbp, NadA, and PorA in the MenB-4C vaccine would not be effective vaccine antigens against *N. gonorrhoeae*. However, a number of other immunogenic proteins seem to have a high level of sequence conservation and may therefore serve as potential vaccine targets. In our study, ~98% of common OMPs present in both *Neisseria* species exhibit between 91% and 100% amino acid sequence similarity within 970 *N. gonorrhoeae* isolates. Approximately 42% of these proteins have been identified in OMV components of the NZ98/254 strain (24, 30, 32, 34). Twelve of these OMPs, including PorB, RmpM, PilQ, OpcA, FetA, Omp85 (BamA), and LbpA, were abundantly and consistently expressed across different OMV lots of the NZ98/254 strain (32), and using bioinformatic analysis, their protein homologs were also identified in publicly available *N. gonorrhoeae* genomes, including FA1090. Of note, while the gene encoding LbpA is expressed in the majority of *N. gonorrhoeae* strains, it is a pseudogene in FA1090 (22). The abundances of other OMPs in the NZ98/254 strain and their potential to serve as candidate vaccine antigens warrant further investigation.

Accurate assessment of protein expression may require optimal protein detection methods. It is worth noting that most OMV vaccines were produced by extraction of vesicles using detergents to remove endotoxin from lipopolysaccharides. This extraction method could lower the content of immunogenic proteins released in OMVs compared to detergent-free methods (55–57). Therefore, the protein composition isolated from OMVs may vary depending on the preparation process. Interestingly, the vaccine antigen FHbp was found exclusively in detergent-free OMVs (30, 56), which may explain its absence in the detergent-extracted OMVs (24, 30, 32, 34) and underscores the importance of choosing proper laboratory methods in these investigations. In regard to cross-protection, one recent finding shows that a MenB vaccine made of native OMVs elicited gonococcal bactericidal antibodies in a mouse model (57).

Quantitative proteomic analyses have been demonstrated to facilitate the identification of surface-expressed gonococcal antigens for vaccine development, such as ubiquitous proteins localized to the cell envelopes and membrane vesicles, including BamA, LptD, TamA, MetQ, and NGO2054 (58, 59). In these studies, assessment of protein subcellular localization was performed using a number of prediction tools, SoSuiGramN, PSORTb, CELLO, and SignalP, of which the last 3 tools and BUSCA were used in our study. While a combination of proteomic and bioinformatic approaches was utilized to identify surface-expressed proteins in the previous studies (58, 59), our data are strictly based on the bioinformatic analysis of bacterial genomes. Among common OMPs identified in our study, PorB, PilQ, Omp85 (BamA), OpcA, LbpA, NspA, MtrE, MetQ, TbpB, and AniA have been experimentally tested as potential gonorrhea vaccine antigens due to their immunogenic and antigenococcal properties *in vitro* and/or in a mouse model (9, 12, 13, 41). Previous findings describe the significance of MetQ (60) and AniA (44–46) as targets for antigenococcal vaccines and therapeutic intervention. Of those previously tested as gonorrhea vaccine candidates, PilQ, Omp85 (BamA), NspA, MtrE, MetQ, and LbpA have been identified within OMVs of the NmB strain NZ98/254 (24, 30, 32, 34) and had $\geq 93\%$ amino acid sequence similarity to *N. gonorrhoeae*, suggesting their potential contribution to mechanisms of cross-protection. On the other hand, PorB and OpcA only showed $\leq 70\%$ amino acid sequence similarity to the respective *N. gonorrhoeae* homologs, which may reduce their cross-protective role against gonorrhea. These 4 OMPs (PorB, PilQ, OpcA, and LbpA) were abundant in the total protein amount quantified across different NZ98/254 OMV lots (32). Interestingly, copper-containing nitrite reductase AniA, the *N. gonorrhoeae* pivotal anaerobic respiration factor, was shown to be expressed only in urethritis isolates from men who have

sex with men (61), providing the rationale for not being detected in the NZ98/254 OMV. Among gonococcal vaccine targets that were not part of our analysis, a monoclonal antibody against the conserved lipooligosaccharide (LOS) 2C7 epitope has been shown to hold promise in preclinical testing (13, 62). Similarly, Liu and colleagues demonstrated that intravaginal immunization of *N. gonorrhoeae*-challenged mice with a mixture of gonococcal OMVs and microencapsulated interleukin-12 resulted in accelerated clearance of infection (63).

Taken together, our bioinformatics approach to examine the genetic similarities between NmB (particularly NZ98/254) and *N. gonorrhoeae* strains contributes to a better understanding of the OMPs present within the MenB-4C vaccine that may be involved in cross-protection and offers valuable insight into antigens of interest to guide next steps for gonorrhea vaccine development. Recognition of gonococcal OMV proteins by MeNZB-like OMV-induced antibodies provides an explanation for the previously observed decrease in gonococcal cases following MeNZB vaccination (22). Investigations of immunogenicity and types of immune responses are required to further investigate the contribution of host defense mechanisms induced by specific OMPs within the MenB-4C OMV against *N. gonorrhoeae* infections.

MATERIALS AND METHODS

Bacterial strain collection. This study included 970 *N. gonorrhoeae* isolates from 27 sites participating in GISP, a national sentinel surveillance system (<https://www.cdc.gov/std/gisp/default.htm>), obtained between 2014 and 2016. These isolates were selected to represent the geographic, temporal, and genetic diversity among the domestic *N. gonorrhoeae* population from an original set of 1,518 isolates previously sequenced for surveillance and for antimicrobial susceptibility studies. The NmB reference strain (MC58) and 3 NmB epidemic strains from New Zealand (NZ98/254), Norway (H44/76), and Cuba (CU385/83) were included as meningococcal reference strains. NZ98/254 belongs to clonal complex 41/44 (CC41/44), while the other 3 NmB strains belong to CC32. The 3 NmB epidemic strains were used in the OMV-containing MenB vaccines; the licensed MenB-4C vaccine contains an OMV from NZ98/254.

DNA sequencing of *N. gonorrhoeae* isolates. Genomic DNA extraction was performed using Qiagen DNeasy kit, MagNA Pure 24 system (Roche), or Promega Wizard DNA extraction kit (Promega). Sequencing libraries were generated using the Nextera XT library preparation kit (Illumina) or NEBNext Ultra DNA library prep kit for Illumina (New England Biolabs), following the manufacturers' instructions or PulseNet protocols (<https://www.cdc.gov/pulsenet/>). High-quality libraries were (paired-end) sequenced on an Illumina MiSeq instrument using V2 reagents at Antibiotic Resistance Laboratory Network laboratories (in Maryland, Washington State, and Texas) and the Hawaii Department of Health or on an Illumina HiSeq instrument using HiSeq 2500 V2 reagents at the CDC Biotechnology Core Facility Branch.

Genome analysis and selection of OMPs. Illumina sequence reads for 970 *N. gonorrhoeae* isolates were trimmed using Cutadapt 1.8 (64) and assembled using SPAdes 3.7.0 (65). Low-coverage contigs relative to the overall genomic coverage were discarded. The assemblies for 4 NmB strains were included in the analysis. All genomes were annotated using the *Neisseria* allele collection from PubMLST (66) to identify and characterize the genomic features found in the assembly. The annotation files were compared to find genes that were common to all 4 NmB strains and found at a frequency of 95% or greater ($n \geq 922$) within *N. gonorrhoeae* genomes. The threshold of 95% represents the "soft-core genes," which includes genes that may otherwise have not been considered due to sequencing, assembly, or annotation errors. These common gene sequences were obtained and translated using custom Python (<https://www.python.org/>) scripts. Any gene sequence without a complete ORF or with premature internal stops was discarded. The resulting collection of protein sequences was used to generate a consensus sequence for each locus for *N. meningitidis* and *N. gonorrhoeae* separately. The consensus protein sequences for NmB and *N. gonorrhoeae* were provided as input to 3 subcellular localization Web servers, Cello2Go (67), BUSCA (68), and PSORTb (69). The molecular function of the proteins was predicted using InterProScan (70) software. Two of these tools, BUSCA and PSORTb, provided signal peptide predictions, while Cello2Go did not. As a result, a third signal peptide prediction software, SignalP (71), was included. The results of these tools were combined and used to identify OMPs of interest.

Analysis of sequence similarity. Unique protein sequences were obtained for the selected OMPs from all 970 *N. gonorrhoeae* and 4 NmB genomes. Protein similarity values were calculated using BLAST (72) for the following comparisons: all 4 NmB strains against all *N. gonorrhoeae* genomes, within all *N. gonorrhoeae* genomes, the NZ98/254 NmB strain against all *N. gonorrhoeae* genomes, and the other three NmB strains against all *N. gonorrhoeae* genomes.

Phylogeny analysis. Protein-based phylogenetic trees were created for selected OMPs. Alignments were created for each OMP using Clustal Omega 1.2 (73), and these alignments were used as input for RAxML 8.2.9 (74) to generate each phylogeny. All phylogenies were visualized and annotated using the Interactive Tree of Life (75) platform.

Data availability. All raw read data have been deposited in the NCBI Sequence Read Archive (SRA) under BioProject accession number [PRJNA317462](https://www.ncbi.nlm.nih.gov/bioproject/PRJNA317462). The accession numbers of the 970 gonococcal genomes used in this study are listed in Table S3 in the supplemental material.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/mBio.01668-19>.

FIG S1, PDF file, 0.5 MB.

TABLE S1, PDF file, 0.2 MB.

TABLE S2, PDF file, 0.1 MB.

TABLE S3, PDF file, 0.2 MB.

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